PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/543, 33/566, 33/58

A1

(11) International Publication Number: WO 98/16833

(43) International Publication Date: 23 April 1998 (23.04.98)

(21) International Application Number: PCT/GB97/02552

(22) International Filing Date: 22 September 1997 (22.09.97)

(30) Priority Data:
9621256.8 11 October 1996 (11.10.96) GB

(71) Applicant *(for all designated States except US)*: XENOVA LIMITED [GB/GB]; 240 Bath Road, Slough, Berkshire SL1 4EF (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: MACALLAN, David [GB/GB]; Xenova Limited, 240 Bath Road, Slough, Berkshire SL1 4EF (GB).

(74) Agents: KEEN, Celia, Mary et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: DOUBLE LABEL IMMUNOASSAY

(57) Abstract

A solid phase assay comprising: (a) providing a solid support on which is immobilised a first component of a binding interaction between the first component and a second component, the first component having a first label which is non–radioactive; (b) contacting the said solid support either (i) with a putative inhibitor of the binding interaction and the second component of the binding interaction, the second component having a second label which is non–radioactive and which is distinguishable from the first label, under conditions under which the binding interaction, in the absence of inhibitor would be expected to occur, or (ii) with a specimen to be assayed for a predetermined analyte, which analyte is the second component of the binding interaction, under conditions under which the binding interaction would be expected to occur if the analyte is present in the specimen; and (c) determining whether, or to what extent, the binding interaction has occurred.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# DOUBLE LABEL IMMUNOASSAY

The present invention relates to an improved solid phase assay.

In conventional solid phase assays, a first component of a binding interaction between the first component and a second component is immobilised on a solid support. Any complex formed by the binding interaction is generally isolated on the solid support and the presence of the isolated complex is determined.

However, in such assays, errors can arise as some of the complex formed by the binding interaction may be lost. Loss of complex may occur if, for some reason, the first component immobilised on the solid support becomes dissociated therefrom. If some of the complex is lost before it can be detected, then subsequent determination of the amount of the complex will give an artificially low result, leading, in some cases, to false assay results.

The present invention seeks to provide a solid phase assay which incorporates an internal control to assess whether, or to what extent, the complex formed by the binding interaction is lost before it is determined.

Accordingly, the present invention provides a solid phase assay comprising:

(a) providing a solid support on which is immobilised a first component of a binding interaction between the first component and a second component, the

first component having a first label which is non-radioactive;

(b) contacting the said solid support either (i) with a putative inhibitor of the binding interaction and the second component of the binding interaction, the second component having a second label which is non-radioactive and which is distinguishable from the first label, under conditions under which the binding interaction, in the absence of inhibitor would be expected to occur, or (ii) with a specimen to be assayed for a predetermined analyte, which analyte is the second component of the binding interaction, under conditions under which the binding interaction would be expected to occur if the analyte is present in the specimen; and

(c) determining whether, or to what extent, the binding interaction has occurred.

The assay of the present invention is advantageous as the first label can function as an internal control to assess whether, or to what extent, the complex formed by the binding interaction is lost before it is determined. If complex is lost before it is determined, the signal generated by the first label will be inhibited.

The binding interaction can be, for example, (a) an interaction between a ligand and a receptor therefor, for example the binding of a cytokine, hormone, neurotransmitter, growth factor or peptide to a receptor therefor, (b) a protein/protein interaction such as the

binding of an antibody to an antigen or a signalling protein interaction, preferably an intracellular signalling protein interaction such as a ras-raf interaction or an interaction between cyclin and cyclin-dependent kinase or (c) an interaction between a DNA sequence and one or more protein which recognises the sequence.

The first component and/or the second component may consist of more than one substance. Thus, for example, the first component may consist of a DNA sequence and the second component may consist of two proteins which can together bind thereto. Either component of a given binding interaction may be the component immobilised on the solid support.

Preferably, the solid support is an optical quality surface, i.e. a surface that has minimal effect on a signal generation process and on a generated signal. The solid support typically comprises polystyrene beads or the wall of a reaction vessel. The solid support may therefore comprise the walls of a 96 well microtitration plate or the walls of a higher density plate. A higher density plate is a plate of the same footprint size as a 96 well microtitration plate having more than 96 wells. Typically, a higher density plate has about 384 wells.

Other suitable solid supports include optical fibres and glass or quartz surfaces such as quartz cuvettes, flat sheets of quartz or glass and quartz or glass rods.

Typically, the assay of the invention includes the

step of immobilising the first component of the binding interaction on the solid support. The first component can be immobilised on the solid support in any convenient manner such as by a protein capture technique. Typically, the first component is covalently or non-covalently bound to a first substance and a second substance, capable of binding to the first substance, is adsorbed onto the solid support. Capture of the first substance by the second substance can then be effected.

Typically the first and second substances are, respectively, biotin and streptavidin, an antigen and an antibody therefor, a glutathione-S-transferase fusion protein and glutathione, or a histidine tagged protein and nickel.

Alternatively, the first component can be passively adsorbed onto the solid support.

In the assay of the present invention, the first and second labels are different. The signals generated by the first and second labels can be distinguished. Thus, the components labelled with the first and second labels can be distinguished.

The first label and/or the second label is preferably fluorometrically detectable. More preferably the first label and/or the second label is fluorescein, calcein, rhodamine or a mutant of green florescent protein. Preferred labels are those which are susceptible of quantitative determination. It is particularly preferred

that at least one, more preferably both, of the first and second labels are lanthanide ion labels such as samarium and europium ion labels.

Typically, a lanthanide ion label is a substantially non-fluorescent lanthanide ion complex covalently bound to the component in question, which is detectable by adding a developer comprising (a) a detergent and (b) a chelating compound with which the lanthanide ion gives fluorescence, thereby dissociating the lanthanide ion from the component and forming a fluorescent lanthanide ion chelate. Further details regarding the developer are given below.

The assay of the invention is typically either an assay for an inhibitor of a binding interaction or an assay of a specimen for a predetermined analyte.

An assay of the invention for an inhibitor of a binding interaction will now be described in more detail. Such an assay is shown in Figure 1.

Typically, an assay for an inhibitor of a binding interaction comprises:

(I) providing a solid support on which is immobilised a first component of a binding interaction between the first component and a second component, the first component having a first label which is non-radioactive;

(II) contacting the said solid support with the second component of the binding interaction and a putative inhibitor of the binding interaction under conditions under

which the binding interaction, in the absence of inhibitor, would be expected to occur, the second component having a second label which is non-radioactive and which is distinguishable from the first label; and

(III) determining whether the putative inhibitor inhibits the binding interaction.

A true positive result, where the putative inhibitor inhibits the interaction between the first and second components, will result in inhibition of the signal generated by the second label. However, the signal generated by the first label will not be inhibited.

A false positive result is generated when the amount of the complex formed by the binding interaction is reduced for any reason other than inhibition of the binding interaction by the putative inhibitor, for example if the putative inhibitor causes the first component to become dissociated from the solid support. In this event, the amount of complex isolated on the solid support in step (III) will be reduced and both the signal generated by the first label and the signal generated by the second label will be inhibited.

False positive results can therefore be detected by means of the present invention.

The conditions in step (II) above will obviously vary depending on the binding interaction but typically comprise incubation at from 10°C to 40°C, preferably at about room temperature, for from 30 minutes to 2 hours, preferably for

about 1 hour.

Step (III) is generally carried out by determining whether, or to what extent, the binding interaction has occurred. This can be done by determining the first and second components of the binding interaction. Typically, step (III) comprises (a) rinsing away unbound substances to isolate on the solid support any complex formed by the binding interaction and (b) determining the presence of the labels in the isolated complex.

If a substantially non-fluorescent lanthanide ion complex, as described above, is used as the first or second label, step (III b) above typically comprises the detection of the label by:

(A) adding to the solid support having the complex isolated thereon, a developer solution comprising (a) a detergent and (b) a chelating compound with which the lanthanide ion gives fluorescence.

(B) allowing the lanthanide ion to dissociate from the component in question and to form a fluorescent chelate; and

(C) determining the fluorescent chelate thereby obtained.

Preferably the lanthanide ion is a samarium or europium ion. Typically, the developer solution further comprises a buffer.

Preferably, the detergent is a non-ionic surfactant.

More preferably it is a polyoxyethylene ether such as an iso-octylphenoxypolyethoxyethanol in which the polyethoxy chain contains about ten ethoxy units (i.e. Triton X-100®). Preferably, the chelating compound is 2-naphthoyltrifluoroacetone, tri-n-octylphosphine oxide or $N^1$-(p-isothiocyanatobenzyl)-diethylenetriamine-$N^1$, $N^2$, $N^3$, $N^4$-tetraacetic acid. Preferably, the buffer is capable of maintaining a pH of from 3 to 4, more preferably about 3.2. Preferably, the buffer is an acetate-phthalate buffer.

Suitable developers are known in the art. An example of a suitable developer is DELFIA® Enhancement solution available from Wallac Oy.

Typically, step (B) is conducted by shaking the solid support with the said complex isolated thereon, in the presence of the developer solution, for from 5 to 20 minutes, for example about 10 minutes.

Greater sensitivity may be obtained by exciting the fluorescent chelates obtained in step (B) by means of a light impulse before determining the fluorescent chelate. During the interval between excitation and detection of the fluorescent chelates, the fluorescence from possible sources of interference decays.

Typically, fluorescent europium chelates are excited from 300 to 500 $\mu$s, preferably about 400$\mu$s, before they are detected. Typically, fluorescent samarium chelates are excited from 40 to 60 $\mu$s, preferably about 50 $\mu$s, before they are detected. Typically, fluorescent terbium chelates

are excited from 80 to 120 μs, preferably about 100μs, before they are detected. Typically, fluorescent dysprosium chelates are detected from 20 to 40 μs, preferably about 30 μs, before they are detected.

Typically, the light impulse is produced by a xenon flash lamp with a flash duration of about 1 μs. The wavelength of the light impulse is typically from 320-340 nm.

Typically the light impulse is generated, and the fluorescence is subsequently detected, in a fluorometer such as the Wallac 1234® fluorometer or the Wallac 1420 Victor Multilabel Counter®.

In an assay of the present invention, which is an assay for an inhibitor of a binding interaction, the effect of a putative inhibitor on two or more different bioaffinity reactions can be investigated in the same assay. Thus, the effect of a putative inhibitor on one or more binding interactions can be investigated simultaneously. In such a multiplex assay, in step (a) of the assay of the invention a labelled first component of each binding interaction is provided on the solid support and in step (b) of the assay of the invention a labelled second component of each binding interaction is contacted with the solid support and the putative inhibitor, the labels for each first component typically being distinguishable from each other and the labels for each second component typically being distinguishable from each

other.

The second component of each binding interaction has a label which can be distinguished from the label of the first component of each binding interaction.

The same label can be used for each second component, in which case any specific inhibition of any of the binding interactions would result in a decrease in the signal generated by the label attached to each second component without a corresponding decrease in any of the signals generated by the labels attached to the first components.

The same label can be used for each first component, in which case any non-specific inhibition of any of the binding interactions would result in a decrease in the signal generated by the label attached to each first component.

If the labels of each of the first components and each of the second components can all be distinguished from one another, it will be possible to assess the effect of the putative inhibitor on each binding interaction.

A particularly preferred assay for an inhibitor of a binding interaction is an assay wherein the first component is tumour necrosis factor $\alpha$ (TNF$\alpha$) and the second component is the p55 or p75 receptor protein. Preferably, both TNF$\alpha$ and the p55 or p75 receptor protein are labelled with a substantially non-fluorescent lanthanide ion complex as described above. Such a complex can be detected by steps (A) to (C), as described above. Preferably, the TNF$\alpha$ is

labelled with a samarium complex and the p55 or p75 protein is labelled with a europium complex.

TNFα is typically immobilised on a solid phase by biotinylation followed by capture with streptavidin which is adsorbed to the solid phase. Preferably, the biotinylated TNFα is immobilised onto plates which are precoated with steptavidin.

It is found that, when TNFα is the first component in the preferred assay described above, non-specific binding is significantly lower than in previous TNFα/receptor protein screening assays. In previous TNFα/receptor protein screen assays, the receptor protein has been immobilised on the solid phase. Non-specific binding consists of all binding between the second component and the solid phase other than binding between the second component and the first component. Non-specific binding can lead to false assay results and a reduction in non specific binding is advantageous.

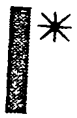
The use of lanthanide labels in the preferred TNFα/receptor protein assay is also advantageous. In previous TNFα/receptor protein assays, [$^{125}$I] labelled TNFα has been detected. It is not possible to determine the [$^{125}$I] labelled TNFα as accurately as the lanthanide labelled component.

Figure 1 shows an assay for the detection of inhibitors of the TNFα and p55 receptor binding interaction. In the assay shown in Figure 1, the first

component of the binding interaction is tumour necrosis factor α (TNFα). The TNFα is covalently bound to biotin, which is adsorbed to streptavidin on the solid phase. The second component of the binding interaction is p55 receptor protein. The TNFα is labelled with a samarium ion label and the p55 receptor protein is labelled with a europium ion label. In Figure 1, the various symbols have the following meanings:

 - Natural product inhibitor

 - Samarium - TNFα - biotin

 - p55-Europium

 - Strepavidin

A - FluoroNunc 96 well plate or 384 well white Nanoplates

The present invention also provides a test kit suitable for an assay for an inhibitor of a binding interaction which test kit comprises:

(a) a solid support; and

(b) labelled first and second components of a binding interaction, as defined above.

Preferably, in the above test kit of the invention,

the first component is immobilised on the solid support.

An assay of the invention which is an assay of a specimen for a predetermined analyte will now be described in more detail.

A solid phase assay, conducted to determine whether a specimen contains a predetermined analyte, typically comprises:

(I) providing a solid support on which is immobilized a first component of a binding interaction between the first component and a second component, the first component having a first label which is non-radioactive;

(II) contacting the said solid support with a specimen to be assayed for a predetermined analyte, which analyte is the second component of the binding interaction, under conditions under which the binding interaction would be expected to occur if the analyte is present in the specimen; and

(III) determining whether the analyte is present in the specimen.

The analyte is typically a substance of diagnostic significance, such as an antibody to a pathogen or an antigen recognised by such an antibody. The first component is typically an antibody or a DNA sequence.

The conditions in step (II) generally comprise incubation at from 18°C to 40°C, preferably at about 37°C. The incubation period will depend on the particular binding

interaction but is typically from 10 to 60 minutes, for example about 30 minutes.

Preferably the specimen is a serum, plasma, urine, saliva, or cerebrospinal fluid specimen, more preferably a serum or plasma specimen.

Step (III) is generally carried out by determining whether, or to what extent, the binding interaction has occurred. This can be done by determining the first component of the binding interaction and the complex formed by the binding interaction.

In step (III), the complex formed by the binding interaction is typically determined by (a) rinsing away unbound specimen components to isolate on the solid support any complex formed by the binding interaction, (b) adding a further reactant which recognises the said complex and which is labelled and (c) determining the label of the further reactant.

The label of the further reactant is distinguishable from the first label. It can be detected by conventional methods. It may be any conventional label such as an enzyme label, a fluorescent label or a radioactive label.

When the analyte is a human antibody, the further reactant can be anti-human IgG conjugated with the enzyme horseradish peroxidase (hrp). The hrp conjugated anti-human IgG binds to complex formed by the binding interaction. Unbound anti-human IgG may be rinsed away and the bound anti-human IgG may be detected by adding a

chromogenic substance containing tetramethyl benzidine (TMB). This reacts with the hrp conjugated to the anti-human IgG to form a blue coloured product. The optical density of the resulting solution is directly proportional to the amount of the complex formed by the binding interaction.

The first label is preferably a substantially non-fluorescent lanthanide ion complex, as described above. It can be detected by steps (A) to (C), as described above.

If the first component should be become dissociated from the solid support, the signal generated by the first label will be inhibited. Thus, one source of false negative results can be detected by means of the present invention.

The present invention also provides a test kit suitable for an assay of a specimen for a predetermined analyte, which test kit comprises a solid support upon which is immobilised a labelled first component of a binding interaction as defined above.

In its broadest aspect, the present invention provides a solid phase assay in which (i) a solid support is provided, upon which a first component of a binding interaction between the first component and a second component is immobilised, (ii) a step is carried out in which the second component may bind to the first component and (iii) whether, or to what extent, the binding interaction has occurred is determined,

wherein the first component is labelled with a non-radioactive label.

Reference Example 1

Lyophilised protein carrier free TNFα was reconstituted in sodium carbonate buffer at pH 9.6. It was then simultaneously labelled with biotin and samarium (Sm) chelate by incubation with biotin-ε-aminocaproic acid-N-hydroxysuccinimide ester and samarium chelated with $N^1$-(p-isothiocyanatobenzyl)-diethylenetriamine-$N^1$, $N^2$, $N^3$, $N^4$-tetraacetic acid for 18 hours at 4°C. The labelled protein was purified by gel filtration chromatography, with an 80% yield. The concentration of the purified labelled protein was estimated to be 80μg/ml. The labelling ratio was calculated to be 0.5:0.7:1 Sm:biotin:TNFα. The labelled purified protein was stabilised with 1 mg/ml Bovine Serum Albumin (BSA) and stored at -70°C.

A Tris-HCl buffered NaCl assay buffer solution (pH 7.8) containing 0.05% $NaN_3$, BSA, Bovine gamma globulins, Tween 40, diethylenetriaminepentaacetic acid and an inert red dye (available from Wallac Oy as DELFIA® assay buffer) was used for diluting the labelled protein. Streptavidin coated 96 well polystyrene plates of good optical quality were used to capture the Sm-TNFα-biotin. Increasing concentrations of Sm-TNFα-biotin were added to streptavidin coated wells (100 μl/well) and incubated for 1 hour at room temperature with shaking.

The plates were then washed six times with 250 μl of a Tris-HCl buffered salt solution wash buffer (pH 7.8) containing Tween 20 and Germall as a preservative (available from Wallac Oy as DELFIA® wash buffer).

100 μl of an enhancement solution containing an iso-octylphenoxypolyethoxyethanol in which the polyethoxy chain contains about ten ethoxy units (Triton X-100®), 2-naphthoyltrifluoroacetone, and tri-n-octylphosphine oxide in acetate-phthalate buffer at pH 3.2 (available from Wallac Oy as DELFIA® enhancement solution) was then added. After shaking for 10 minutes to allow dissociation enhancement of the lanthanides, the samarium signals were measured in the Wallac 1234® fluorometer. The results are shown in Table 1.

**Table 1**

Titration of Sm-TNFα-Biotin Coating Concentration

| | Sm-TNFα-Biotin Signal | |
|---|---|---|
| ng/well | Average | Standard Error of Mean |
| 0 | 239 | 9 |
| 0.5 | 384 | 16 |
| 1 | 581 | 18 |
| 2 | 922 | 11 |
| 5 | 2588 | 98 |
| 10 | 6749 | 72 |
| 20 | 16884 | 165 |
| 30 | 25196 | 1234 |
| 40 | 36380 | 652 |
| 50 | 44532 | 323 |
| 60 | 56427 | 775 |
| 70 | 62657 | 612 |

Binding was observed to increase linearly with increasing concentrations of Sm-TNFα-biotin.

Reference Example 2

Lyophilised protein carrier free recombinant p55 receptor protein was reconstituted in sodium carbonate buffer at pH 9.6. It was then labelled with europium (Eu) chelate by incubation with europium chelated with $N^1$-(p-isothiocyanatobenzyl)-diethylenetriamine $N^1$, $N^2$, $N^3$, $N^4$-tetraacetic acid for 18 hours at 4°C. The labelled protein

was purified by gel filtration chromatography, with an 80% yield. The concentration of the purified labelled protein was estimated to be 80μg/ml. The labelling ratio was calculated to be 2.1:1 Eu:p55. The labelled purified protein was stabilised with 1mg/ml BSA and stored at -70°C.

An assay buffer as described in Reference Example 1 was used for diluting the labelled protein. Streptavidin-coated 96 well plates to which Sm-TNFα-biotin was bound were prepared as in Reference Example 1 using 20ng Sm-TNFα-biotin per well. These plates were then washed three times with 250 μl of a wash buffer as described in Reference Example 1. The purified p55-Eu was then added at various concentrations (100μl/well) and incubated at room temperature with shaking for 2 hours.

The plates were then washed six times with 250 μl of the above wash buffer and 100μl of the enhancement solution described in Reference Example 1 was added. After shaking for 10 minutes to allow dissociation enhancement of the lanthanides, the samarium and europium signals were measured in a Wallac 1234® fluorometer. Non-specific binding was measured by repeating the experiment using streptavidin-coated plates to which no Sm-TNFα-biotin was bound. The results are shown in Table 2.

Table 2

Saturation of p55-Eu Binding to Sm-TNFα-Biotin

| Total Binding - Eu Signal | | | Non-specific Binding - Eu signal | | |
|---|---|---|---|---|---|
| [p55-Eu] (M) | Average | Standard Error of Mean | [p55-Eu] (M) | Average | Standard Error of Mean |
| 0 | 930 | 42 | 0 | 261 | 16 |
| 1.00E-11 | 3861 | 102 | 1.00E-11 | 253 | 14 |
| 3.00E-11 | 10325 | 205 | 3.00E-11 | 237 | 7 |
| 1.00E-10 | 29693 | 799 | 1.00E-10 | 274 | 22 |
| 3.00E-10 | 87774 | 3415 | 3.00E-10 | 241 | 5 |
| 1.00E-09 | 231898 | 5889 | 1.00E-09 | 308 | 34 |
| 3.00E-09 | 519891 | 8226 | 3.00E-09 | 313 | 25 |
| 1.00E-08 | 786687 | 44825 | 1.00E-08 | 378 | 73 |
| 3.00E-08 | 1086753 | 37327 | 3.00E-08 | 362 | 24 |
| 1.00E-07 | 1249340 | 16469 | 1.00E-07 | 604 | 79 |
| 3.00E-07 | 1349215 | 84382 | 3.00E-07 | 921 | 40 |

| Sm-TNFα-Biotin signal | | |
|---|---|---|
| [p55-Eu] (M) | Average | Standard Error of Mean |
| 0 | 16357 | 201 |
| 1.00E-11 | 14834 | 462 |
| 3.00E-11 | 15812 | 469 |
| 1.00E-10 | 15328 | 473 |
| 3.00E-10 | 15889 | 629 |
| 1.00E-09 | 16259 | 376 |
| 3.00E-09 | 16738 | 382 |
| 1.00E-08 | 16274 | 712 |
| 3.00E-08 | 16459 | 762 |
| 1.00E-07 | 16442 | 642 |
| 3.00E-07 | 17037 | 574 |

Total binding for p55-Eu was found to saturate with a maximum signal of from 1,200,000 to 1,400,000 europium counts. The non-specific binding was very low at 300 - 2,000 europium counts over this range of p55-Eu concentrations. Therefore this assay had a 700-4,000 fold window (assay window = total binding/non-specific binding).

Half maximal binding at 600,000-800,000 europium counts was achieved at a p55-Eu concentration of from 5 to 10 nM. This would be equivalent to the Kd value for the binding of TNFα to p55 protein. However, the exact concentration of p55 at which half maximal binding was achieved could not be accurately determined. Therefore, a displacement experiment using unlabelled p55 to compete with the p55-Eu (see below) was performed to calculate Kd.

The level of Sm-TNFα-biotin bound in this experiment gave the expected signal of from 15,000 to 17,000 samarium counts and was uniform across the plate.

Example 1

Inhibition of p55-Eu binding to Sm-TNFα-biotin by unlabelled p55 over the range $10^{-11}$ - $10^{-6}$ M was determined. A fixed final concentration of 7nM p55-Eu was used as this represents the approximate Kd value obtained in Reference Example 2. The assay was conducted in the same way as in Reference Example 2 except that, as explained above, a fixed concentration of p55-Eu was used and unlabelled p55

was added, together with the europium labelled p55, at various concentrations (100µl/well final volume). The results are shown in Table 3.

**Table 3**

Inhibition of p55-Eu by Unlabelled p55

| p55-Eu Signal | | | Sm-TNFα-Biotin Signal | | |
|---|---|---|---|---|---|
| [p55] (M) | Average | Standard Error of Mean | [p55] (M) | Average | Standard Error of Mean |
| 1.00E-11 | 701602 | 15820 | 1.00E-11 | 14844 | 343 |
| 3.00E-11 | 715996 | 14948 | 3.00E-11 | 15145 | 337 |
| 1.00E-10 | 691250 | 23129 | 1.00E-10 | 15101 | 513 |
| 3.00E-10 | 640688 | 30800 | 3.00E-10 | 15298 | 737 |
| 1.00E-09 | 520921 | 6481 | 1.00E-09 | 15623 | 238 |
| 3.00E-09 | 244664 | 12082 | 3.00E-09 | 15064 | 446 |
| 1.00E-08 | 78544 | 4101 | 1.00E-08 | 15341 | 552 |
| 3.00E-08 | 24181 | 1113 | 3.00E-08 | 14949 | 485 |
| 1.00E-07 | 8451 | 337 | 1.00E-07 | 15537 | 542 |
| 3.00E-07 | 3870 | 885 | 3.00E-07 | 14615 | 441 |
| 1.00E-06 | 1578 | 57 | 1.00E-06 | 14333 | 304 |

The results in Table 3 show 50% inhibition at 1.9nM. This gives a close approximation to the Kd for p55-Eu binding. However, a more accurate indication of the Kd can be obtained by calculating the Ki from the $IC_{50}$ (the concentration at which 50% of the binding is inhibited) using the Cheng-Prusoff equation:

$Ki=IC_{50}/(1+L/Kd)$ where L = p55-Eu concentration.

In this case L should equal the Kd. Thus:

$Ki=IC_{50}/2$

Therefore, the Ki for p55 inhibition of p55-Eu is found to be 0.95 nM which is equivalent to literature values for the Kd of the p55 receptor (0.3 - 1.0 nM). The control samarium signal was as expected in the assay at around 15,000 samarium counts.

Reference Example 3

In Example 1 and Reference Example 2, a 2 hour incubation period was used for binding of the p55-Eu to the Sm-TNFα-biotin. To determine an optimum incubation time, a time course of binding of p55-Eu to Sm-TNFα-biotin at a fixed final concentration of 7nM was performed with 15 minute time points. The assay was conducted in the same way as in Reference Example 2 except that, as explained above, the concentration of p55-Eu was fixed at 7nM and the incubation time was varied. The results are shown in Table 4.

**Table 4**

Time Course for p55 Binding

| p55-Eu Signal | | | | | | |
|---|---|---|---|---|---|---|
| Time | Total Binding | | Non-specific Binding | | Sm-TNFα-Biotin Signal | |
| (min) | Average | Standard Error of Mean | Average | Standard Error of Mean | Average | Standard Error of Mean |
| 15 | 634846 | 29296 | 312 | 16 | 16227 | 455 |
| 30 | 747059 | 18120 | 324 | 22 | 17314 | 478 |
| 45 | 773710 | 12773 | 340 | 43 | 17681 | 211 |
| 60 | 791242 | 14181 | 316 | 30 | 17597 | 456 |
| 75 | 805514 | 15279 | 361 | 25 | 17585 | 439 |
| 90 | 809398 | 26920 | 367 | 27 | 17618 | 564 |
| 105 | 798321 | 29524 | 382 | 40 | 16755 | 687 |
| 120 | 799861 | 20818 | 366 | 34 | 16543 | 536 |

The results in Table 4 demonstrate that p55-Eu binding to Sm-TNFα-biotin is rapid and has reached equilibrium by 60 minutes.

Reference Example 4

A Sm-TNFα-biotin complex was prepared as described in Reference Example 1. The labelled protein was diluted with the Tris-HCl buffered NaCl assay buffer described in Reference Example 1. 384 well white polystyrene plates of good optical quality were coated with 40 μg/ml streptavidin overnight at 4°C. These streptavidin coated plates were used to capture the Sm-TNFα-biotin. The plates were first

blocked using 2% BSA and then increasing concentrations of Sm-TNFα-biotin were added to the streptavidin coated wells (total volume 50 μl/well). Incubation then took place for 1 hour with shaking.

The samarium ions were determined as described in Reference Example 1. The results are shown in Table 5.

**Table 5**

Titration of Sm-TNFα-Biotin Coating Concentration

| | Sm-TNFα-Biotin Signal | |
|---|---|---|
| ng/wells | Average Sm-TNFα-Biotin signal | Standard error of mean |
| 0 | 342 | 16 |
| 1.875 | 4359 | 153 |
| 3.75 | 7931 | 532 |
| 7.5 | 13988 | 402 |
| 15 | 21638.75 | 838 |
| 30 | 27964 | 2083 |
| 60 | 36012.75 | 205 |
| 120 | 47356 | 261 |
| 240 | 53276.5 | 894 |
| 480 | 57301.25 | 3311 |

Reference Example 5

Purified p.55 receptor protein labelled with europium chelate was prepared as described in Reference Example 2. Streptavidin coated 384 well plates to which Sm-TNFα-biotin was bound were prepared as in Reference Example 5 using 50

μl of 120 ng/ml Sm-TNFα-biotin. The plates were then washed three times with a wash buffer as described in Reference Example 1. The purified p.55-Eu was then added at various concentrations (total volume 50μl/well). Incubation took place at room temperature with shaking for 2 hours.

The europium ions were determined as in Reference Example 2. The results are shown in Table 6.

**Table 6**

Saturation of p55-Eu binding to the Sm-TNFα-Biotin

| [p55-Eu] μM | Average Eu signal | Standard of error of mean |
|---|---|---|
| 0 | 3035 | 209 |
| 0.0003 | 157064 | 19461 |
| 0.0007 | 361555 | 19371 |
| 0.0014 | 671915 | 13578 |
| 0.0027 | 1049680 | 35934 |
| 0.0054 | 1492000 | 79303 |
| 0.0109 | 2021490 | 131363 |
| 0.0210 | 2154510 | 99581 |
| 0.0437 | 2441790 | 111060 |
| 0.0874 | 2638210 | 117080 |
| 0.1749 | 2946350 | 149617 |
| 0.3498 | 3075610 | 205654 |

Total binding for p55-Eu was found to saturate with a maximum signal of approximately 3,000,000 europium counts, with non-specific binding at 3000 europium counts. This gave an assay window of 1000 fold (assay window = total

binding/non-specific binding). Half-maximal binding at 1,500,000 europium counts was achieved at a p55-Eu concentration of approximately 5.3nM. This would be equivalent to the Kd value for the binding of TNFα to p55. However, the exact concentration of p55 at which half-maximal binding was achieved could not be accurately determined. Therefore, a displacement experiment using unlabelled p55 was performed to calculate the Kd, as in Example 1.

Example 2

Inhibition of p55-Eu binding to Sm-TNFα-biotin by unlabelled p55 was determined. The assay was conducted in the same way as in Reference Example 5 except that a fixed concentration of 120 ng/ml Sm-TNFα-biotin was used and unlabelled p55 was used, together with the europium labelled p55, at various concentrations. The results are shown in Table 7.

**Table 7**

Inhibition of p55-Eu binding by unlabelled p55

| p55-Eu Signal | | |
|---|---|---|
| [p55] ($\mu$M) | Average p55-Eu signal | Standard error of mean |
| 3.55 E-7 | 3579560 | 7463 |
| 1.77 E-6 | 3586380 | 6944 |
| 8.8 E-6 | 3583760 | 6177 |
| 4.4 E-5 | 3552030 | 34637 |
| 2.2 E-4 | 3441980 | 10076 |
| 1.1 E-3 | 3236970 | 63011 |
| 5.5 E-3 | 1775460 | 60112 |
| 2.78 E-2 | 207993 | 18330 |
| 1.39 E-1 | 46732 | 1425 |
| 5.56 E-1 | 20260 | 716 |

The results in Table 7 show 50% inhibition at a p55 concentration of 4.12nM. The Cheng-Prusoff equation was used to determine the p55 Ki and hence p55-Eu Kd from the p55 $IC_{50}$, as shown in Example 1.

Ki = $IC_{50}/(1+L/Kd)$ where L = ligand concentration which in this case was 2 x estimated Kd or 10.6nM.

Therefore, the Ki for the p55 inhibition of p55-Eu for the 384 well format was found to be 1.37nM which is close to the literature quoted values.

Reference Example 6

An optimum incubation time for binding of the p55-Eu to the Sm-TNFα-Biotin was determined by carrying out an assay in the same way as in Reference Example 5 except that the concentration of p55-Eu was fixed at 2nM and the incubation time was varied. The results are shown in Table 8.

**Table 8**

Time course for p55 binding

| p55-Eu Signal | | | | |
|---|---|---|---|---|
| Time (mins) | Total Binding | Total Binding | Non-specific binding | Non-specific binding |
| | Average | Standard error of mean | Average | Standard error of mean |
| 0 | 0 | 0 | 0 | 0 |
| 15 | 278389 | 42071 | 33296 | 436 |
| 30 | 400000 | 54733 | 63159 | 829 |
| 45 | 470000 | 28043 | 24084 | 1523 |
| 60 | 469828 | 30365 | 63845 | 551 |
| 75 | 466837 | 28661 | 73256 | 711 |
| 120 | 448198 | 43163 | 25063 | 2112 |
| 160 | 448830 | 29365 | 63669 | 873 |

Reference Example 7

The use of different assay plates was investigated for both the 96 and 384 well assay formats. In both cases clear, white and black plates were evaluated.

96 well plates were coated with streptavidin as in Reference Example 4, but using a total volume of 100μl in each reagent and wash step. Sm-TNFα-biotin at 120ng/ml (100μl) was then added and incubated for one hour at room temperature with shaking. The plates were then washed three times with a wash buffer as described in Reference Example 1 prior to the addition of 7.7nM p55-Eu (100μl total volume). After a further 1 hour incubation the plates were washed six times with the above wash buffer and 100μl of the enhancement solution described in Reference Example 1 was added. After shaking to allow dissociation enhancement of the lanthanides, the plates were mixed and the samarium and europium signals were determined using a Wallac Victor 1420 reader. Clear, black and white plates were handled identically.

Clear, black and white 384 well plates were handled in the same manner, but with total volumes in each reagent addition stage of 50μl.

Tables 9, 10 and 11 show the data for the 96 well plates. Tables 12, 13 and 14 show the data for the 384 well plates.

**Table 9**

Data obtained using clear 96 well plates

in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 36905 | 5979 | 72 |
| Average europium count for NSBs | 515 | 134 | |
| Average samarium count for Totals | 2834 | 122 | 21 |
| Average samarium count for NSBs | 134 | 13 | |

**Table 10**

Data obtained using black 96 well plates

in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 29946 | 7538 | 133 |
| Average europium count for NSBs | 226 | 32 | |
| Average samarium count for Totals | 1584 | 277 | 90 |
| Average samarium count for NSBs | 18 | 3 | |

**Table 11**

Data obtained using white 96 well plates in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 585471 | 36952 | 72 |
| Average europium count for NSBs | 8099 | 234 | |
| Average samarium count for Totals | 38654 | 2506 | 97 |
| Average samarium count for NSBs | 243 | 6 | |

The TOTAL values are those obtained adding both Sm-TNFα-biotin and p55-Eu in the assay. The NSB (non-specific binding) refer to those values obtained using p55-Eu alone. The concentrations used were 120ng/ml SM-TNFα-biotin and 7.7nM p55-Eu.

**Table 12**

Data obtained using clear 384 well plates

in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 52175 | 4966 | 109 |
| Average europium count for NSBs | 477 | 55 | |
| Average samarium count for Totals | 3369 | 334 | 18 |
| Average samarium count for NSBs | 188 | 38 | |

**Table 13**

Data obtained using black 384 well plates

in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 37670 | 11970 | 241 |
| Average europium count for NSBs | 156 | 33 | |
| Average samarium count for Totals | 2744 | 483 | 164 |
| Average samarium count for NSBs | 17 | 8 | |

Table 14

Data obtained using white 384 well plates

in the TNFα dual labelled assay

| | Average count | Standard deviation from the mean | Assay window |
|---|---|---|---|
| Average europium count for Totals | 294887 | 33255 | 228 |
| Average europium count for NSBs | 1292 | 655 | |
| Average samarium count for Totals | 16586 | 1795 | 151 |
| Average samarium count for NSBs | 110 | 19 | |

The TOTAL values are those obtained adding both Sm-TNFα-biotin and p55-Eu in the assay. The NSB (non-specific binding) refer to those values obtained using p55-Eu alone. The concentrations used were 120ng/ml SM-TNFα-biotin and 7.7nM p55-Eu.

The data in Tables 9, 10, 11, 12, 13 and 14 show that this assay system works well and provides significant counts and assay windows (assay window = total counts/NSB counts) in clear, white and black plates for both the 96 and 384 well formats.

## CLAIMS

1. A solid phase assay comprising:

(a) providing a solid support on which is immobilised a first component of a binding interaction between the first component and a second component, the first component having a first label which is non-radioactive;

(b) contacting the said solid support either (i) with a putative inhibitor of the binding interaction and the second component of the binding interaction, the second component having a second label which is non-radioactive and which is distinguishable from the first label, under conditions under which the binding interaction, in the absence of inhibitor would be expected to occur, or (ii) with a specimen to be assayed for a predetermined analyte, which analyte is the second component of the binding interaction, under conditions under which the binding interaction would be expected to occur if the analyte is present in the specimen; and

(c) determining whether, or to what extent, the binding interaction has occurred.

2. An assay according to claim 1 wherein the first and second labels are fluorometrically detectable.

3. An assay according to claim 1 or 2 wherein the binding interaction is an interaction between a ligand and a receptor therefor.

4. An assay according to claim 1 or 2 wherein the binding interaction is a protein/protein interaction.

5. An assay according to claim 1 or 2 wherein the binding interaction is an interaction between a DNA sequence and one or more protein which is capable of binding thereto.

6. An assay according to any one of the preceding claims wherein the first label and/or the second label is a substantially non-fluorescent lanthanide ion complex covalently bound to the component in question, which is detectable by adding a developer comprising (a) a detergent and (b) a chelating compound with which the lanthanide ion gives fluorescence, thereby dissociating the lanthanide ion from the component and forming a fluorescent lanthanide ion chelate.

7. An assay according to any one of the preceding claims in which the effect of a putative inhibitor on two or more binding interactions can be investigated simultaneously, wherein in step (a) a labelled first component of each binding interaction is provided on the solid support and in step (b) a labelled second component of each binding interaction is contacted with the solid support and the putative inhibitor, the labels for each first component being distinguishable from the labels for each second component.

8. An assay according to claim 7 wherein

each first component has the same label.

9. An assay according to claim 7 or 8 wherein each second component has the same label.

10. An assay according to any one of the preceding claims wherein the first component is tumour necrosis factor α (TNFα) and the second component is p55 or p75 receptor protein.

11. A test kit suitable for an assay according to any one of the preceding claims which test kit comprises:

(a) a solid support; and

(b) labelled first and second components as defined in claim 1.

12. A test kit according to claim 11 wherein the first component is immobilised on the solid support.

13. A process for identifying an inhibitor of a binding interaction, which process comprises:

(I) carrying out steps (a), (b)(i) and (c) as specified in claim 1; and, if the putative inhibitor does not inhibit the binding interaction;

(II) repeating (I) using a different putative inhibitor in step (b)(i).

14. An inhibitor of a binding interaction identified by the process of claim 13.

15. An assay according to claim 1 wherein the specimen to be assayed for a predetermined analyte is a serum, plasma, urine, saliva or cerebrospinal fluid

specimen.

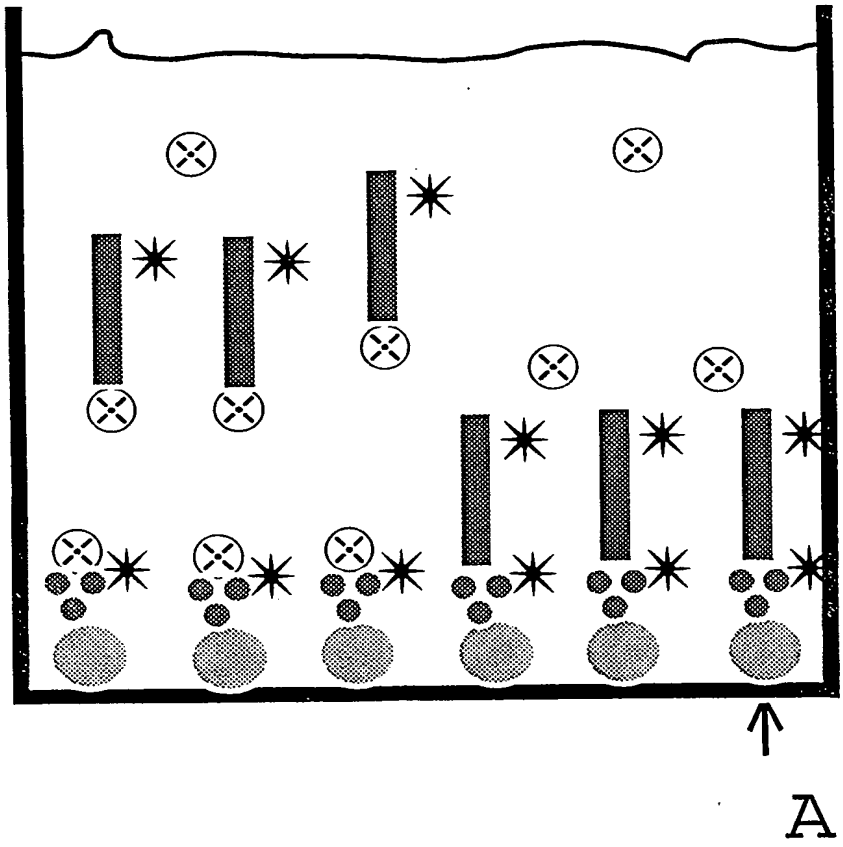
16. An assay according to claim 1 or 15 wherein the analyte is an antibody to a pathogen or an antigen recognised by such an antibody.

17. A test kit suitable for an assay of a specimen for a predetermined analyte, which test kit comprises a solid support upon which is immobilised a labelled first component of a binding interaction as defined in any one of the preceding claims.

18. A solid phase assay in which (i) a solid support is provided, upon which a first component of a binding interaction between the first component and a second component is immobilised, (ii) a step is carried out in which the second component may bind to the first component and (iii) whether, or to what extent, the binding interaction has occurred is determined,

wherein the first component is labelled with a non-radioactive label.

FIGURE 1



# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02552

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N33/566 G01N33/58

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 0 271 974 A (EKINS ROGER PHILIP) 22 June 1988<br>see claims 1-9,11,12<br>see page 11, line 7 - page 12, line 2;<br>figure 1 | 1-18 |
| X | PATENT ABSTRACTS OF JAPAN<br>vol. 016, no. 276 (P-1374), 19 June 1992<br>& JP 04 072564 A (MITSUBISHI KASEI CORP),<br>6 March 1992,<br>see abstract | 1-18 |
| A | EP 0 093 613 A (SYVA CO) 9 November 1983<br>Whole document | 1-18 |
| A | WO 91 00519 A (TRITON BIOSCIENCES INC) 10 January 1991<br>Whole document | 1-18 |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

| Date of the actual completion of theinternational search | Date of mailing of the international search report |
|---|---|
| 15 December 1997 | 05/01/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Routledge, B |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02552

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP 0271974 A | 22-06-88 | AU 614935 B | 19-09-91 |
| | | AU 7755987 A | 24-02-88 |
| | | CA 1284620 A | 04-06-91 |
| | | DE 3784465 A | 08-04-93 |
| | | DK 183088 A | 05-04-88 |
| | | EP 0318491 A | 07-06-89 |
| | | FI 92881 C | 10-01-95 |
| | | FI 92881 B | 30-09-94 |
| | | WO 8801058 A | 11-02-88 |
| | | JP 1503405 T | 16-11-89 |
| | | KR 9610696 B | 07-08-96 |
| | | US 5171695 A | 15-12-92 |
| EP 0093613 A | 09-11-83 | US 4533629 A | 06-08-85 |
| | | US 4540659 A | 10-09-85 |
| | | AU 560535 B | 09-04-87 |
| | | AU 1411083 A | 10-11-83 |
| | | BR 8302286 A | 03-01-84 |
| | | CA 1193193 A | 10-09-85 |
| | | DE 3377744 A | 22-09-88 |
| | | HK 96090 A | 23-11-90 |
| | | IN 156680 A | 12-10-85 |
| | | JP 1857426 C | 27-07-94 |
| | | JP 58206966 A | 02-12-83 |
| | | JP 2651060 B | 10-09-97 |
| | | JP 5209886 A | 20-08-93 |
| | | US 5342759 A | 30-08-94 |
| | | US 4843000 A | 27-06-89 |
| | | US 4849338 A | 18-07-89 |
| | | US 5432057 A | 11-07-95 |
| WO 9100519 A | 10-01-91 | DE 69008178 D | 19-05-94 |
| | | DE 69008178 T | 06-10-94 |
| | | EP 0479929 A | 15-04-92 |
| | | US 5506109 A | 09-04-96 |